Docket No.: 050229-0377

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Customer Number: 20277
David HILDEBRAND, et al.	:	Confirmation Number: 4235
Application No.: 10/622,774	:	Group Art Unit: 1638
Filed: July 21, 2003	:	Examiner: Vinod KUMAR

For: RECOMBINANT STOKESIA EPOXYGENASE GENE

DECLARATION OF DAVID HILDEBRAND UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, DAVID HILDEBRAND, declare as follows:

1. I am one of the named inventors on the above-captioned patent application directed to the recombinant *Stokesia Epoxygenase* gene. I have Ph.D in Plant Breeding and Genetics from the University of Illinois. My curriculum vitae is attached hereto.

2. I am familiar with the prosecution of the above-captioned and the statements made by the Examiner in Office Action mailed November 28 2006 and with respect to the claims at issue being rejected under 35 U.S.C. § 112 first paragraph for allegedly failing to reasonably provide enablement for a method of using a transformed host cell other than bacterial or plants cells with a nucleic acid molecule encoding a protein of SEQ ID No. 2.

3. Experiments in which epoxy fatty acid formation was observed in yeast and plant seed host cells when transformed with the *Stokesia* epoxygenase gene were conducted under my supervision as evidenced by the enclosed article "Expression of a *Stokesia laevis* epoxygenase gene", Phytochemistry (2004) pages 1-8 attached herewith, which is co-authored by me.

4. As demonstrated in the enclosed article an increase of epoxy fatty acid formation was observed in yeast and plant seed host cells transformed with the *Stokesia laevis* epoxygenase gene.

5. I hereby declare that all statements made herein are of my own knowledge are true and that all statements made on information and are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Signed this 21 day February, 2007

David Hildebrand, PhD.

David Floyd Hildebrand

VITA

Rank: Professor, Department of Agronomy, University of Kentucky

Birth: March 12, 1955 - Bethesda, Maryland

Education:

B.S. in Agronomy,	University of Maryland (1977)
B.S. in Chemistry,	University of Maryland (1977)
M.S. in Plant Breeding and Genetics,	University of Illinois (1980)
Ph.D. in Plant Breeding and Genetics,	University of Illinois (1982)

Professional Career:

Junior Fellowship in biomedical research with US-National Institutes of Health (NIH), 1973-1977.

Research Assistant in Plant Physiology, University of Maryland, 1977.

Graduate Research Assistantships, University of Illinois, 1977-1981.

Graduate Fellowships, University of Illinois, 1979, 1980.

Japanese Society for the Promotion of Science Fellowship for work at Kyoto University Research Institute for Food Science, February 1982-August, 1982.

Assistant Professor, University of Kentucky, August 1982-1988.

Associate Professor, University of Kentucky, April 1988-

Professor, University of Kentucky, April 1997-

Member of the Crop Science, Plant Physiology and Nutritional Sciences graduate programs at the Univ. Kentucky.

Current Research Focus:

My research program emphasizes the general area of plant biochemistry and genetics and the application of biotechnology to crop improvement with particular emphasis on food, lipid and oil quality, new uses of agricultural commodities and plant pest defense. This research involves the investigation of metabolic pathways and the identification, isolation, cloning and manipulation by plant genetic engineering of agriculturally important genes. The major research thrust is the understanding and manipulation of fatty acid metabolism and triglyceride synthesis. We are modifying triglycerides of oilseeds, with emphasis on soybeans, for improved edible and industrial quality. Towards this end we have been involved in extensive research in collaboration with Glenn Collins on the establishment of regeneration and transformation systems for soybeans and the coupling of transformation to regeneration. Work in my lab during the 1997-98 period helped lead to a significant reduction in the time needed for the soybean somatic embryogenesis transformation/regeneration protocol. For improved edible quality we are changing the ratios of the mix of vegetable oil fatty acids by reducing both the saturated and polyunsaturated fatty acid percentages with corresponding increases in monounsaturated fatty acids. We were the first to show altered fatty acid metabolism including > 60% reduction in saturated fatty acid levels in genetically engineered plant tissues. These studies will result in healthier and more stable vegetable oil products with greater acceptability to consumers. To further these goals we have cloned several Δ12 desaturase cDNAs. For industrial uses we are tailoring the triglycerides towards much higher tri-unsaturated fatty acid level which would make vegetable oils much more valuable in several industrial products such as "drying oils". Unique ω3 desaturases have been partially characterized which will not only be useful for increasing industrial utility of vegetable oils, but also for increased ω3 fatty acids needed in healthy diets and ω3 are the principal components of photosynthetic membranes.

We are also are working toward developing oilseed oils high in epoxy fatty acids which will greatly increase their value for a large number of industrial products. Epoxy fatty acids are examples of "oxylipins", or oxygenated products of fatty acids. Certain plants, which accumulate high levels of epoxy fatty acids in seed oil, have an enzyme not present in major oil seeds including soybeans known as epoxygenase. We have thoroughly characterized epoxygenases from some high epoxide accumulating plants including *Vernonia galamensis* and *Euphorbia lagascae* biochemically. This has lead to the surprising discovery that different plant families have evolved different mechanisms for epoxy fatty biosynthesis with some using diiron desaturase-like oxygenases and others using P450 monooxygenases. We have used the biochemical information we have accumulated to clone several epoxygenase cDNAs.
Another major thrust of my research program is the detailed understanding of oxylipin formation in plant tissues. Most plant tissues form a range of oxylipins. Some oxylipins are very important in the flavor and aroma and therefore general quality of plant derived foods. We and others find that several oxylipin molecules increase dramatically with plant stresses such as desiccation, mechanical damage such as caused by insect feeding and pathogen invasion. Some of these induced oxylipins, including aldehydes, epoxides, traumatin and jasmonic acid have been demonstrated by us and others to be important in plant pest defense and defense signaling systems. As our studies are improving the understanding and control of oxylipin formation, we have initiated work towards the manipulation of their formation in genetically engineered plants for improved food quality and enhanced disease and insect resistance. Some oxylipins, including certain alcohols, aldehydes and esters, are among the most important food and beverage flavoring ingredients and there is considerable interest in improved natural sources of many of these oxylipins. We have made progress in developing plants as bioreactors for production of these molecules. By over-expressing a key branch enzyme of the oxylipin pathway, allene oxide synthase, in transgenic tobacco under control of a tightly regulated promoter, we have been able to shift production of one group of oxylipins to another.

Some oxylipins actually have a very negative impact on flavor and aroma of some food products particularly those derived from soybeans. There is currently considerable interest in increasing the soybean consumption for health reasons and our research is leading to improved methods of processing soybean-derived foods. We have found that use of soybeans with different lipoxygenases can lead to improved nutritional, rheological and baking quality of fortified breads together with Kwako Addo in food science. We have also made progress in understanding how to control peroxidation of polyunsaturated fatty acids of membrane lipids in order to slow postharvest deterioration of vegetables such as broccoli.

I am currently taking the principal responsibility for teaching Plant Biochemistry, one of the core courses for the Plant Physiology, Biochemistry and Molecular Biology program. I believe this is a very important component of plant science related graduate programs and is very useful to our graduates. My aim is to have this course benefit our graduate students maximally. Towards this goal I have developed an extensive web site and numerous handouts clarifying and reinforcing key principals of plant biochemistry. The handouts alone will be useful reference materials for our graduates.

Professional Society Membership:

American Association for the Advancement of Science
American Society of Agronomy
Crop Science Society of America
Plant Molecular Biology Association
Gamma Sigma Delta Honor Society
American Oil Chemists' Society
American Society of Plant Physiologists

PROFESSIONAL RECOGNITION

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Elected Secretary/Treasurer of the Biotechnology Division of the AOCS International 1998 – 2001; vice-chair 2001 – 2004 and chairperson of the Biotechnology Division of the AOCS 2004 - present
Associate Editor JAOCS 1999 – present
NIH Review Panel member May 2000, March 2001
Chair of the American Oil Chemists' Society's international committee on Genetically Modified Organisms

TEACHING RESPONSIBILITIES

Major course development and teaching

AGR/ACS 562 - Advanced Genetics - Redeveloped the course content taught the complete course (3 h) four times: in the Fall of 1983; Fall of 1984; Spring of 1986 and Fall of 1987.

AGR697A - Developed this course - Introduction to Biotechnology. 1 credit hour. Taught in Spring 1989 and Fall, 1990.

BCH/PLS/PPA 609 - Plant Biochemistry - Reorganized and assumed principal responsibility (22/29 lectures) for teaching fall 1996 and spring 1998, 1999, 2000 (and probably every year for at least the next several years). Developed an extensive web site (http://www.uky.edu/~dhild/biochem/welcome.html) to enhance Plant Biochemistry instruction.

Received a grant from the Teaching and Learning Center (TLC) for Innovative Teaching Projects: "Enhanced computer-based Plant Biochemistry instruction" for the Spring 2000. The funds were used to hire a student to assist in developing enhanced computer-based instructional tools for the students. This included developing greatly embellished illustrations to clarify biochemical principals, computer animations and interactive computer-based problems. The web-based interactive programs automatically indicated to the students whether they understood how important aspects of fundamental biosynthetic pathways functioned. For some examples visit the following URLs:

http://www.uky.edu/~dhild/biochem/23/lect23.html
http://www.uky.edu/~dhild/biochem/24/lect24.html
http://www.uky.edu/~dhild/biochem/16/lect16.html

Miscellaneous lectures and teaching

GS 600C - co-organizer of this Special Topics in Molecular & Cellular Genetics Seminar series in the fall 1987.

AGR623 - Advanced Plant Physiology - Taught six lectures on Introduction to Biotechnology in the Spring of 1989.

AGR799 - Crop Science Seminar. 1 credit hour. Coordinator in Fall, 1989 and Spring, 1990.

AGR630 - Techniques in Plant Physiology Lab Course - Taught a section on Gas Chromatography and Lipid Analysis Techniques in the Summer of 1990.

Taught a section in the course Food Chemistry (FSC 434G) on lipids.

AGR773 - Plant Physiology Seminar. Organizer in Fall 1986 and 1992. Also have advised students who give topic seminars.

Guest lecturer in Foods and Nutrition course and seminar, Spring 1994 and 1995.

NS 651/NSF 772 - gave two lectures spring 1996, 1997 and 1998.

CNU 606, Molecular Biological Applications in Nutrition- gave two lectures spring 1996.

SERVICE

International: Chaired a session on Novel Production Technology of New Agricultural Products at the International Workshop on Life Science in Production and Food-Consumption of Agricultural Products hosted by the Japanese government in October 1993. Biotechnology Division Officer of the AOCS 1996 - 1997. Member of student scholarship committee of AOCS 1996 -. Secretary/treasurer of the Biotechnology Division of the AOCS 1998 -. American Society of Plant Biologists, ASPB, UK campus contact. Chair of the American Oil Chemists' Society's international committee on Genetically Modified Organisms 2000 – present. Chairperson of the AOCS Biotechnology Division Lifetime Achievement Award selection committee in 2000 and 2001. Chaired the organizing committee of the biotechnology symposia (> 60 invited presentations) of the May 2002 AOCS meetings in Montreal. Organized a symposium on plant lipid biochemistry at the 2003 AOCS meeting in Kansas City and co-organizer of the joint symposia of the joint Japanese Oil Chemist Society (JAOCS) and the AOCS in Cincinnati in 2004. Session chair and organizer of the general biotechnology symposium at the 2005 AOCS meetings in Salt Lake City in 2005.

National: Chaired the Metabolism section at the National Plant Physiology Meetings in 1991 and 1992. Organized a symposium on "Genetic Improvement of Oilseeds" at the American Oil Chemists' Society National Meetings in 1996, Co-chaired a symposium on "Utilization of Vegetable Oils for Non-Food Uses" in 1997 and on "Advances in Genetic Modification of Soybean Oil" in 1998. Organized Three sessions of the Soy 2000 8th Bienial Conference of the Cellular and Molecular Biology of the Soybean on 1.) Tissue Culture and Transformation, 2.) Metabolic Engineering and Value Added Traits and 3.) Genomics in August, 2000. Member of the United Soybean Board (better bean initiative)

BBI working group and participate in their annual meetings in St. Louis every year 1998 - 2005.

Regional: Southern American Oil Chemists' Society technical committee. Member and periodic participant in the Kentucky Academy of Sciences. Participated in and made a presentation at an all day Agent Training Workshop on Biotechnology at the Rural Development center in Somerset Sept. 2000. Gave two presentations at the Women in Agriculture Workshop in Louisville in the Fall 2000 and at the Southern States regional meeting in February, 2001. Made a presentation at the National Science Teacher's Association meetings in Louisville in 2003. Participated in the Kentucky "Critical Technologies" K-12 teaching needs workshop 2004.

College and University: Manned the phones for an evening and made some follow-up calls at other times for the Undergraduate student recruitment Phonathon. Actively participate in the undergraduate biotechnology program. Served on the Graduate School's Committee Grant evaluation committee in November, 1992 and 1994 and the major equipment grant committee November, 2001. Member of the Senate Advisory Faculty Code Committee 1996 to 1998. Co-chair of the Biotechnology Research and Education Initiative (BREI) Committee. BREI web site: http://www.ca.uky.edu/brei/ Served on the committee that developed the Plant Science dedication program June 2000. Co-organized and presented a display at the college Academic showcase program Sept. 2000. Put together a display for the BREI booth at the 2001 - 2002 College of Agriculture Roundups. Chaired the Gamma Sigma Delta graduate student awards committee Spring 2002. Chaired the undergraduate Agricultural Biotechnology (ABT) program review committee in Spring 2002 (see Teaching Portfolio for activities and reports). Put together and manned a display for the BREI booth at the 2003 and 2004 College of Agriculture Roundup Events and a display on genetic engineering of grain crops for the 2003 Agronomy field day. Co-organized a CSI-biotech 4-H camp for two days in June 2004 in Greenup Co. Helped organize a GMO forum with the Good Foods Coop in Oct. 2004. Represented life sciences for UK at the Kentucky Linking Leaders Event: Discussion of "Kentucky Survey of Critical Technologies" meeting at NKU in Sept. 2004. Presentation on plant genetic engineering at a meeting of KY science teachers in Nov. 2004. Helping organize college P-12 science educational outreach.

Departmental: Co-chair of Crop Science Seminar Committee Fall 1989 and Spring 1990. Chaired the Plant Physiology Seminar Committee Fall 1986 and 1992. Member scholarship and safety committees 1996. Put together a display for the Agronomy booth at the 1998 College of Agriculture Roundup.

Regular Ad hoc reviewer for NSF, USDA, NIH and DOE competitive grants programs. Also, American Soybean Association (United Soybean Board).

Served on an NIH panel June, 2000 and May 2001 and 2002.

Associate Editor J. Am. Oil Chem. Soc. 1999 to present.

Invited to write a number of book chapters and edit a book.

Invited to write a section for the Encyclopedia of Applied Plant Sciences on "Genetics of Crop Improvement; Primary Metabolism - Lipids" by Academic Press 2002.

Regular Ad hoc reviewer for the following journals:

Plant Physiology
Plant Molecular Biology
Physiology Plantarum
Crop Science
Cereal Chemistry
Journal of American Oil Chemist's Society
Lipids
Hortscience
Journal of Agriculture and Food Chemistry
Journal of Food Science
Phytochemistry

Periodic reviewer for many other journals including The Plant Cell, Nature Biotechnology and the Journal of Biological Chemistry (JBC). Reviewed several plant biochemistry, physiology and biotechnology texts.

ABSTRACTS PRESENTED: 117

RESEARCH PUBLICATIONS:

Kachroo, A, J. Shanklin, E. Whittle, L. Lapchyk, D. Hildebrand and P. Kachroo. 2007. The Arabidopsis stearoyl-acyl carrier protein-desaturase family and the contribution of leaf isoforms to oleic acid synthesis. Plant Molec. Biol. 63: 257-271.

Yu, K., C. T. McCracken, Jr., R. Li and David Hildebrand. 2006. Diacylglycerol acyltransferases from *Vernonia* and *Stokesia* prefer substrates with vernolic acid. Lipids 41: 557-566.

Fukushige, H. and D.F. Hildebrand 2005. A simple and efficient system for green note compound biosynthesis by use of certain lipoxygenase and hydroperoxide lyase sources. Journal of Agricultural and Food Chemistry 53:6877-6882.

Afitlhile, M.M., H. Fukushige, and D. Hildebrand. 2005. Allene Oxide Synthase and Hydroperoxide lyase Product Accumulation in *Artemisia* species. Plant Science 169: 139-146.

Fukushige, H., C. Wang, T.D. Simpson, H.W. Gardner and D.F. Hildebrand. 2005. Purification and identification of linoleic acid hydroperoxides generated by soybean lipoxygenases 2 and 3. Journal of Agricultural and Food Chemistry 53: 5691 -5694.

Fukushige, H. and D.F. Hildebrand. 2005. Watermelon (*Citrullus lanatus*) hydroperoxide lyase greatly increases C_6 aldehyde formation in transgenic leaves. Journal of Agricultural and Food Chemistry 53: 2046 -2051.

Afitlhile, M.M., H. Fukushige, and D. Hildebrand. 2005. A defect in glyoxysomal fatty acid β–oxidation reduces jasmonic acid accumulation in Arabidopsis. Plant Physiol. Biochem. 43: 603-609.

Hatanaka, T., R. Shimizu, and D. Hildebrand. 2004. Expression of a *Stokesia laevis* epoxygenase gene. Phytochemistry 65: 2189-2196.

Kachroo, A., S.C. Venugopal, L. Lapchyk, D. Falcone, D. Hildebrand, and P. Kachroo. 2004. Oleic acid levels regulated by glycerolipid metabolism modulate defense gene expression in Arabidopsis. Proc. Natl. Acad. Sci. -US 101: 5152-5157.

Afitlhile, M.M., H. Fukushige, and D. Hildebrand. 2004. Labeling of major plant lipids and jasmonic acid using [1-^{14}C] lauric acid. Phytochemistry 65: 2679-2684.

Nandi, A., P. Kachroo, H. Fukushige, D.F. Hildebrand, D.F. Klessig and J. Shah. 2003. Ethylene and jasmonic acid signaling pathways affect NPR1-independent expression of defense genes without impacting resistance to *Pseudomonas syringae* and *Peronospora parasitica* in the Arabidopsis *ssi1* mutant. Molec. Plant-Microbe Interactions 16: 588-599.

Kachroo, A., L. Lapchyk1, H. Fukushige, D. Hildebrand, D. Klessig and P. Kachroo. 2003. Plastidal fatty acid signaling modulates salicylic acid- and jasmonic acid mediated defense pathways in the Arabidopsis *ssi2* mutant. Plant Cell 15: 2952-2965.

Kachroo, P., A. Kachroo, L. Lapchyk, D. Hildebrand and D.F. Klessig. 2003. Restoration of defective cross talk in *ssi2* mutants: Role of salicylic acid, jasmonic acid, and fatty acids in *SSI2*-mediated signaling. Molec. Plant-Microbe Interactions 16: 1022-1029.

Hildebrand, D. and K. Yu. 2003. GENETICS OF CROP IMPROVEMENT; PRIMARY METABOLISM – ACYL LIPIDS. pp 464 - 477 In: Encyclopedia of Applied Plant Sciences, D. Murphy, ed., Elsevier Science B.V.

Kim, C.Y., H. Yang, E.T. Thorne, H. Fukushige, Y. Liu, W. Gassmann, D. Hildebrand, R. E. Sharp and S. Zhang. 2003. Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. Plant Cell 15: 2707-2718.

Moon, H. and D.F. Hildebrand. 2003. Effects of Proliferation, Maturation, and Desiccation Methods on Germination and Conversion of Soybean Somatic Embryos. In Vitro Cell. Dev. Biol. 39: 623-628.

He, Y., H. Fukushige, D. Hildebrand and S. Gan. 2002. Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. Plant Physiol. 128: 876–884.

Wang, C., K.P.C. Croft and D.F. Hildebrand. 2001. α-Naphthaleneacetic acid induces the expression of seedling lipoxygenases in soybean immature embryo cotyledons. Plant Cell Rep. 20: 85-91.

Hildebrand, D.F., M. Afitlhile and H. Fukushige. 2000. Regulation of oxylipin synthesis. Biochemical Society Transactions 28:851-853.

Moon, H., J. Hazebroek and D.F. Hildebrand. 2000. Changes in fatty acid composition in plant tissues expressing a mammalian Δ9 desaturase. Lipids 35: 471-479.

Wang, C., C.A. Zien, R. Welti, D.F. Hildebrand and X. Wang. 2000. Involvement of phospholipase D in wound-induced accumulation of jasmonic acid in *Arabidopsis*. The Plant Cell 12:2237-2246.

Hage, T.G., C. Seither and D. Hildebrand. 2000. Isolation of two cDNAs from *Vernonia galamensis* (Cass.) Less. Encoding a Microsomal Oleate Desaturase (FAD2) (Accession No AF188263 and AF188264) and Functional Expression in *Saccharomyces cerevisiae*. Plant Physiol. 122: 1457.

Wang, C. U. Jarlfors and D.F. Hildebrand. 1999. Regulation and subcellular localization of auxin-induced lipoxygenases. Plant Sci. 148:147-153.

Wang, C., S. Avdiushko and D.F. Hildebrand. 1999. Overexpression of a cytoplasm-localized allene oxide synthase promotes the wound-induced accumulation of jasmonic acid in transgenic tobacco. Plant Molecular Biol. 40:783-793.

Wang, C. K.P.C. Croft, U. Jarlfors and D.F. Hildebrand. 1999. Subcellular localization studies indicate that lipoxygenase 1-6 are not involved in lipid mobilization during soybean germination. Plant Physiol. 120:227-236.

Avdiushko. S.A., G.C. Brown, D.L. Dahlman and D.F. Hildebrand. 1997. Methyl jasmonate exposure induces insect resistance in cabbage and tobacco. Environ. Entomol. 26:642-654.

Zhuang, H., M.M. Barth and D.F. Hildebrand. 1997. Temperature influenced lipid peroxidation and deterioration in broccoli buds during postharvest storage. Post Harvest Biol. Technol. 10:49-58.

Pfeiffer, T.W., D.F. Hildebrand and E.C. Lacefield. 1997. Registration of Camp-lx_2 soybean germplasm line with small seed and null for lipoxygenase-2. Crop Sci. 37:1986-1987.

Cumbee, B., D.F. Hildebrand and K. Addo. 1997. Soybean flour lipoxygenase isozyme effects on wheat flour dough rheological and breadmaking properties. J. Food Sci. 62:281-294.

Liu, W., R.S. Torisky, K.P. McAllister, S. Avdiushko, D. Hildebrand and G.B. Collins. 1996. A mammalian desaturase gene lowers saturated fatty acid levels in transgenic soybean embryos. Plant Cell, Tissue & Organ Culture 47:33-42.

Budziszewski, G., K.P.C. Croft and D.F. Hildebrand. 1996. Uses of biotechnology in modifying plant lipids. Lipids 31:557-569.

Zhuang, H., T.R. Hamilton-Kemp, R.A. Andersen and D.F. Hildebrand. 1996. The impact of alteration of polyunsaturated fatty acid levels on C_6-aldehyde formation of *Arabidopis thaliana* leaves. Plant Physiol. 111:805-812.

Trawatha, S.E., D.M. TeKrony and D.F. Hildebrand. 1995. Relationship of soybean seed quality to fatty acid and C6-aldehyde levels during storage. Crop Sci. 35:1415-1422.

Avdiushko, S., K.P.C. Croft, G.C. Brown, D.M. Jackson, T.R. Hamilton-Kemp and D.F. Hildebrand. 1995. Effect of volatile methyl jasmonate on the oxylipin pathway in tobacco, cucumber, and Arabidopsis. Plant Physiol. 109:1227-1230.

Brown, G.C., G.L. Prochaska, D.F. Hildebrand, G.L. Nordin and D.M. Jackson. 1995. Green leaf volatiles inhibit conidial germination of the entomopathogen *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae). Environ. Entomol. 24:1637-1643.

Zhuang, H., D.F. Hildebrand and M.M. Barth. 1995. Senescence of broccoli buds is related to changes in lipid peroxidation. J. Agric. Food Chem. 43:2585-2591.

Liu, W., D.F. Hildebrand and G.B. Collins. 1995. Auxin-regulated changes of fatty acid content and composition in soybean zygotic embryo cotyledons. Plant Science 106:31-42.

Grayburn, W.S. and D.F. Hildebrand. 1995. Progeny analysis of tobacco that express a mammalian Δ9 desaturase. J. Am. Oil Chem. Soc. 72:317-321.

Kasu, T., G.C. Brown and D.F. Hildebrand. 1995. Formation of lipoxygenase products in *Phaseolus vulgaris* L. leaves as a response to twospotted spider mite (Acari: Tetranychidae) feeding and their effect on spider mite populations. J. Kan. Entomol. Soc. 68:27-34.

Trawatha, D.M. TeKrony and D.F. Hildebrand. 1995. Soybean lipoxygenase mutants and seed longevity. Crop Sci. 35:862-868.

Choi, D., R.M. Bostock, S. Avdiushko and D.F. Hildebrand. 1994. Lipid-derived signals that discriminate wound- and pathogen-responsive isoprenoid pathways in plants: Methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. Proc. Natl. Acad. Sci. USA 91:2329-2333.

Andersen, R.A., T.R. Hamilton-Kemp, D.F. Hildebrand C.T. McCracken, Jr., R.W. Collins and P.D. Fleming. 1994. Structure-antifungal activity relationships among volatile C_6 and C_9 aliphatic aldehydes, ketones and alcohols. J. Agric. Food Chem. 42:1563-1568.

Shibata, D., A. Slusarenko, R. Casey, D. Hildebrand and E. Bell. 1994. Gene nomenclature: Lipoxygenases. Plant Molec. Biol. Rep. 12:S41-S42.

Zhuang, H., M.M. Barth and D.F. Hildebrand. 1994. Packaging influence total chlorophyll, soluble protein, fatty acid composition and lipoxygenase activity in broccoli florets. J. Food Sci. 59:1171-1174.

Kasu, T., G.C. Brown and D.F. Hildebrand. 1994. Application of fatty acids to elicit lipoxygenase-mediated host-plant resistance to twospotted spider mites (Acari: Tetranychidae) in *Phaseolus vulgaris* L. Environ. Entomol. 23:437-441.

Liu, W., D.F. Hildebrand, P.J. Moore and G.B. Collins. 1994. Expression of desiccation-induced and lipoxygenase genes during the transition from the maturation to the germination phases in soybean somatic embryos. Planta 194:69-76.

Avdiushko, S.A., X.S. Ye, J. Kuc and D.F. Hildebrand. 1994. Lipoxygenase is an abundant protein in cucumber exudates. Planta 193:349-357.

Trawatha, S.E., D.M. TeKrony and D.F. Hildebrand. 1993. Lipoxygenase activity and C6-aldehyde formation in comparison to germination and vigor during soybean seed development. Crop Sci. 33:1337-1344.

Hildebrand, D.F., G.C. Brown, D.M. Jackson and T.R. Hamilton-Kemp. 1993. Effects of some leaf-emitted volatile compounds on aphid population increase. J. Chem. Ecol. 19:1875-1887.

Kenward, K.D., M. Altschuler, D. Hildebrand and P.L. Davies. 1993. Accumulation of type I fish antifreeze protein in transgenic tobacco is cold-specific. Plant Molec. Biol. 23:377-385.

Pfeiffer, T.W., D.F. Hildebrand and J.H. Orf. 1993. Inheritance of a lipoxygenase-1 allozyme in soybean. Crop Sci. 33:691-693.

Avdiushko, S.A., X.S. Ye, D.F. Hildebrand and J. Kuc. 1993. Induction of lipoxygenase activity in immunized cucumber plants. Physiol. Molec. Plant Pathol. 42:83-95.

Andersen, R.A., P.D. Fleming, T.R. Hamilton-Kemp, and D.F. Hildebrand. 1993. pH changes in smokeless tobaccos undergoing nitrosation during prolonged storage: Effects of moisture, temperature and duration. J. Agric. Food Chem. 41:968-972.

Addo, K., D. Burton, M.R. Stuart, H.R. Burton and D.F. Hildebrand. 1993. Soybean flour lipoxygenase isozyme mutant effects on bread dough volatiles. J. Food Sci. 58:583-586.

Deng, W., T.R. Hamilton-Kemp, M.T. Nielsen, R.A. Andersen, G.B. Collins and D.F. Hildebrand. 1993. Effects of six-carbon aldehydes and alcohols on bacterial proliferation. J. Agric. Food Chem. 41:506-510.

Grayburn, W.S., G.B. Collins and D.F. Hildebrand. 1992. Fatty acid alteration by a Δ9 desaturase in transgenic tobacco tissue. Bio/Technology 10:675-678.

Zhuang, H.,, T.R. Hamilton-Kemp, R.A. Andersen and D.F. Hildebrand. 1992. Regulation of C_6-aldehyde formation during soybean leaf development. Plant Physiol. 100:80-87.

Hamilton-Kemp, T.R., C.T. McCracken, Jr., J.H. Loughrin, R.A. Andersen and D.F. Hildebrand. 1992. Effects of some natural volatile compounds on the pathogenic fungi *Alternaria alternata* and *Botrytis cinerea*. J. Chem. Ecol. 18:1083-1091.

Pfeiffer, T.W., D.F. Hildebrand and D. Tekrony. 1992. Agronomic performance of soybean lipoxygenase isolines. Crop Sci. 32:357-362.

Hildebrand, D.F. 1992. Altered Fatty Acid Metabolism in Plants. Food Technol. 46:71-74.

Deng, W., W.S. Grayburn, T.R. Hamilton-Kemp, G.B. Collins and D.F. Hildebrand. 1992. Expression of soybean embryo lipoxygenase 2 in transgenic tobacco tissue. Planta 187:203-208.

Dahmer, M.L., D.F. Hildebrand and G.B. Collins. 1992. Comparative protein accumulation patterns in soybean somatic and zygotic embryos. In Vitro Cell Dev. Biol 28P:106-114.

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Expression of a *Stokesia laevis* epoxygenase gene

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Abstract

Epoxy fatty acids have a number of important uses and there is interest in enzymes catalyzing their synthesis from renewable sources. Both cytochrome P450 monooxygenases and divergent forms of di-iron desaturases are known to produce epoxy fatty acids in plants. Degenerate primers based on conserved sequences of Δ^{12} desaturase-like genes led to the isolation of an epoxygenase gene from *Stokesia laevis*. The cDNA is 1.4 kb and it encodes 378 amino acids. The similarities of this gene at the amino acid sequence level with epoxygenases of *Vernonia* and *Crepis*, and the Δ^{12} desaturases of soybean, *FAD2-1* and *FAD2-2*, are 84%, 69%, 49%, and 55%, respectively. When the vector, pYES2, was used to transform yeast, epoxy fatty acid formation was observed in the cells. The effects of electron donors in the yeast expression system were tested but cytochrome b_5 and cytochrome b_5 reductase genes from *Arabidopsis thaliana* co-expressed with the epoxygenase had little effect on vernolic acid accumulation in the yeast. Finally, this gene, driven by a seed-specific phaseolin promoter, was cloned into a TDNA-vector and transferred into *Arabidopsis* plants. The results showed that T_2 seeds of transgenic *Arabidopsis* expressing the *Stokesia* gene accumulated vernolic acid but no vernolic acid was detected in control plants. Northern blot analysis indicates this *S. laevis* epoxygenase gene is expressed mainly in developing seeds and no transcript was detected in leaves or roots.

Keywords: *Stokesia laevis*; Asteraceae; Epoxygenase; Vernolic acid

1. Introduction

Epoxy fatty acids have many industrial applications such as in drying oils. Currently, epoxidized soybean and linseed oils e.g., linoleic acids, are produced by introducing an epoxy group across the double bonds of polyunsaturated fatty acids. This is a costly process and it would likely be more economical if the biosynthetic reactions in oilseed themselves converted the polyunsaturated fatty acids into epoxy fatty acids. There was no known way to produce a commercial oilseed that accumulates epoxy fatty acids by conventional breeding and genetics. However, certain genotypes of several plant species accumulate high levels of epoxy fatty acids in the seed oil. Epoxy fatty acids, like vernolic (E-12,13-epoxyoctadeca-E-9-enoic) **2** and coronaric (E-9,10-epoxyoctadeca-E-12-enoic) acids, have been found as a component of the seed oil of species represented by a number of plant families, such as Asteraceae, Euphorbiacea, Pnagraceae, Dipsacaceae, and Valerianacea (Smith, 1970). One of the highest known natural accumulators of vernolic acid **2** is *Vernonia galamensis* in which it can constitute 80%, of triglyceride fatty acids (Perdue, 1989, Pascal and Correal, 1992, Bafor et al., 1993, Thompson et al., 1994). *Stokesia laevis* another Asteraceae species native to the southeastern US has seed oil containing 60–70% vernolic acid **2**.

Many plants are known to possess enzymes that transform unsaturated fatty acids into epoxy fatty acids (Blee, 1998, Gardner, 1991). The process by which the seeds of certain species of *Vernonia*, *Stokesia* and *Eu-*

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phorbia synthesize the epoxy fatty acid, vernolic acid **2**, appears to be due to an enzyme not present in major commercial oilseeds. Biochemical studies by Bafor et al. (1993) indicate that developing seeds of these plants contain an enzyme known as an epoxygenase which converts linoleic acid **1** into vernolic acid **2** in a one step reaction (Fig. 1). Although many plants including soybeans have enzymes such as lipoxygenase and peroxygenase that can produce epoxy fatty acids in some disease resistance reactions (Blee, 1998), they do not have mechanisms for accumulation of epoxytriglycerides in their seeds unlike epoxy triglyceride accumulators such as *Vernonia* and *Stokesia* mentioned above (Bafor et al., 1993, Hildebrand et al., 2002).

The original studies on epoxy fatty acid synthesis and accumulation in oilseeds by Bafor et al. (1993) indicated that the epoxy fatty acid that accumulates in seeds of *Euphorbia lagascae*, vernolic acid **2**, is synthesized by an epoxygenase enzyme which is a P450 monooxygenase enzyme. It was assumed that other epoxy fatty acid accumulators such as *Vernonia* similarly synthesized epoxy fatty acids by P450 monooxygenase enzymes. However, studies by our group (10–12) indicated that Δ^{12} fatty acid desaturase-like enzymes are responsible for vernolic acid **2** biosynthesis in epoxy fatty acid accumulators of the Asteraceae such as *Vernonia*, *Crepis* and *Stokesia* unlike the Euphorbiacea epoxide accumulator, *E. lagascae*, which our studies confirmed relied on a P450 monooxygenase (Seither, 1996, Seither et al., 1996, 1997). Lee et al. (1998) and Hitz (1998) confirmed this with cloning cDNAs encoding epoxygenases from *Crepis palaestina* and *V. galamensis* that they found to be members of a growing family of Δ^{12} fatty acid desaturase-like analogs that also includes hydroxylases, acetylenases and conjugases (Cahoon et al., 2001). Cahoon et al. (2002) cloned and characterized the epoxygenase from *E. lagascae* showing that it is indeed a cytochrome P450 monooxygenase.

Kinney et al. (1998) developed a transgenic soybean expressing *Vernonia* epoxygenase, but the content of epoxy fatty acid in seed oil was only about 8%. This suggests that high concentrations of epoxy fatty acids in membrane lipids might be toxic and additional enzymes are needed for selective accumulation of epoxy fatty acids in triacylglyceride.

We here report cloning an epoxygenase gene from *S. laevis* and the expression of this gene in yeast and plants.

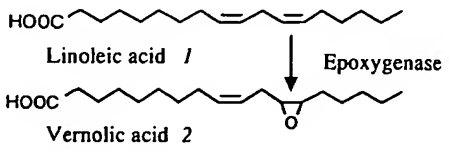

Fig. 1. Vernolic acid 2 synthesis from linoleic acid 1.

2. Results and discussion

2.1. cDNA cloning

The genes of the plant Δ^{12} desaturase family are highly homologous. To design degenerate primers, conserved regions in epoxygenases and an acetylenase but different from desaturases were chosen. As a result of RT-PCR and RACE, the epoxygenase cDNA cloned from *S. laevis* was fully sequenced. It is 1.4 kb, the ORF is 1134 bp and encodes 378 amino acids. The GenBank Accession Number is AY462108. The deduced amino acid sequences of these genes have high similarities to Δ^{12} desaturases of many other plant species. The amino acid similarities of the protein encoded by this gene with epoxygenase of *V. galamensis* and *Crepis*, Δ^{12} desaturase of soybean, FAD 2-1 and FAD 2-2 are 84%, 68%, 49% and 55%, respectively.

Lenman et al. (1998) reported a partial sequence of an epoxygenase-like gene of *V. galamensis*. It is quite different from *Vernonia* epoxygenase cloned by Hitz (1998). We also cloned an epoxygenase gene from *V. galamensis*. As the results of sequencing, this cDNA was slightly different from the gene cloned by Hitz (1998). They have a six amino acid difference, one gap and the similarity is 98.4%. These differences are mainly at the N-terminal region. It is suggested that *V. galamensis* has more than one isozyme of epoxygenase.

The phylogenetic tree analysis of the amino acid sequences of the proteins encoded (Fig. 2) suggests the plant Δ^{12} desaturase gene family includes epoxygenase, hydroxygenase, acetylenase and conjugase. *Stokesia* epoxygenase is closer to *Vernonia* epoxygenase than *Crepis* epoxygenase. Although they all belong Asteraceae family, *Crepis* is in a different tribe from the one which *Vernonia* and *Stokesia* are in. This fact may explain their genetic distance of epoxygenase genes.

2.2. Gene testing in yeast

The cDNA from *S. laevis* was first tested in a yeast, *Saccharomyces cerevisiae*, expression system. However no vernolic acid **2** was detected by our first approach. In a control culture without linoleic acid **1** feeding, linoleic acid **1** was also not detected. It is known that yeast cells accumulate oleic acids and this system works for Δ^{12} desaturases testing. Therefore, it was suggested that this gene was not a Δ^{12} desaturase. We tried to modify the yeast expression system using *Vernonia* epoxygenase gene as a positive control, by administering ^{14}C-labeled linoleic acid **1** as a substrate. The transformed yeast also did not produce any vernolic acid **1**. Assays of microsomes isolated from the transgenic yeast were likewise negative. It seemed very difficult to achieve epoxygenase expression in yeast despite the effective interaction with the electron donors, cytochrome b_5 and cytochrome b_5

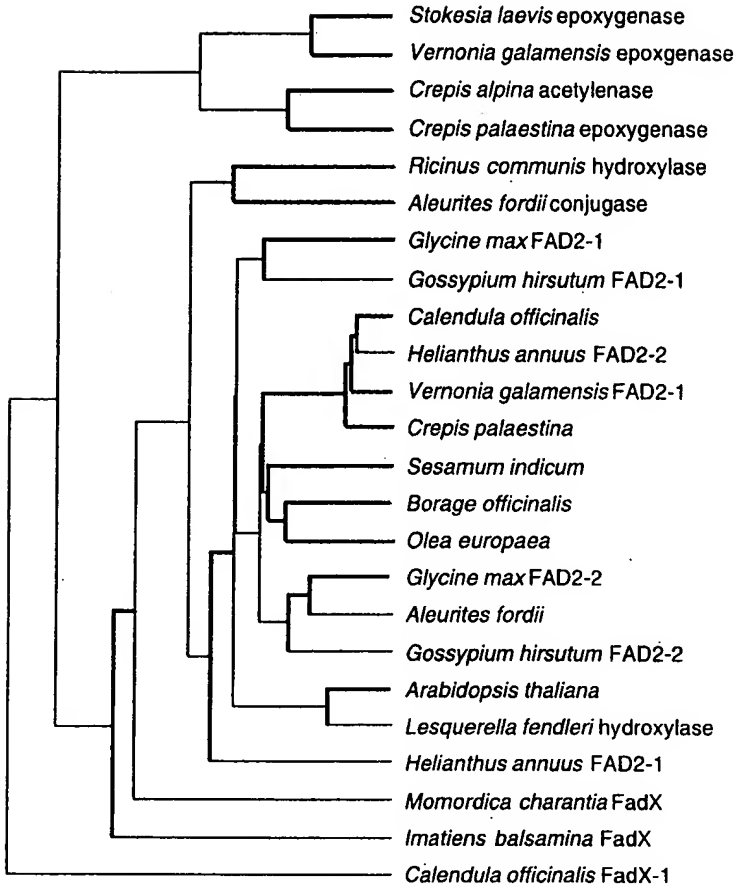

Fig. 2. Phylogenetic tree analysis of plant microsomal Δ^{12} desaturases, epoxygenases, hydroxygenases, conjugases and an acetylenase. The phylogenetic tree represents results from the unweighted pair-group method of the arithmetic average (UPGMA) clustering analysis of amino acid sequences obtained using Kimura distance method (Kimura, 1983). The entries with plant names only are microsomal Δ^{12} desaturases. The GenBank accession numbers used for the analysis were: *Crepis alpina* acetylanase, Y16285; *Crepis palaestina* epoxygenase, Y16283; *Ricinus communis* hydroxylase, U22378; *Aleurites fordii* conjugase, AF525535; *Glycine max* FAD2-1, L43920; *Gossypium hirsutum* 1, X97016; *Calendula officinalis*, AF343065; *Helianus annuus* FAD2-2, AF251842; *Vernonia galamensis* desaturase FAD2-1, AF188263; *Crepis palaestina*, Y16284; *Sesamum indicum*, AF192486; *Borage officinalis*, AF074324; *Olea europea*, AY083163; *Glycine max* FAD2-2, L43921; *Aleurites fordii*, AF525534; *Gossypium hirsutm* 2, Y10112; *Arabidopsis thaliana*, L26296; *Lesquerella fendleri* hydroxylase, AF016103; *Helianthus annuus* FAD2-1, U91341; *Momordica charantia* conjugase, AF182521; *Impatiens balsamina* conjugase, AF182520; *Calendula officinalis* conjugase, AF343064; and *Vernonia galamensis* epoxygenase, United States Patent: 5,846,784.

reductase with various homologous Δ^{12} desaturases tested with the same vector in yeast (Hage et al., 2000; data not shown).

To clarify the effects of electron donors in a yeast expression system, cytochrome b_5 and cytochrome b_5 reductase genes from *A. thaliana* were introduced into yeast as a second approach. Unexpectedly, we detected vernolic acid **2** and epoxystearic acid in both yeast lines transformed with the *Stokesia* epoxygenase gene with (0.64%) or without (0.55%) *A. thaliana* cytochrome b_5 and cytochrome b_5 reductase genes, but not in control yeast cells (Fig. 3) in media supplemented with linoleic acid. An average of 0.64% of vernolic acid **2** in yeast expressing *Stokesia* epoxygenase gene together with *A. thaliana* cytochrome b_5 and cytochrome b_5 reductase genes is slightly higher than in yeast expressing the *Stokesia* epoxygenase gene only. However, this difference is not significant. Dyer et al. (2002) reported conjugated fatty acids formation in yeast transformed with tung conjugase gene also using the pYES vector. These facts suggest that the vector used, pYES2, led to successful production of these unusual fatty acids in yeast cells.

2.3. Gene testing in plants

To verify the enzymatic activity of the gene product in plants, the protein was expressed in seeds of *A. thaliana*. In seeds of transgenic *Arabidopsis*, vernolic acid was detected with GC–MS, but not in control plants transformed with the empty vector (Fig. 4). Sixteen individual T_2 seeds were tested by GC. The contents of

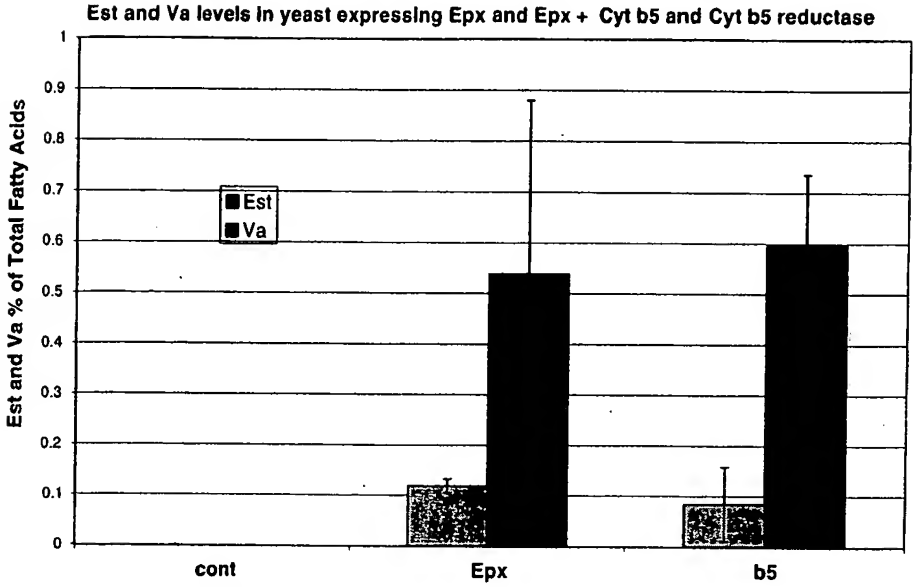

Fig. 3. GC analysis of epoxy stearic (Est) and vernolic (Va) acids in Yeast expressing *S. laevis* epoxygenase (EPX) or *S. laevis* EPX + *Arabidopsis* Cytochrome b_5 and Cytochrome b_5 reductase (EPX + b_5, $b_5$red). The numbers in the figure showed % of epoxy stearic and vernolic acids of total fatty acids. Yeast were transformed with the expression vector pYES2 and pESC lacking a cDNA insert for the vector control (CTR), pYES-St EPX together with pESC (EPX) and pYES-St EPX together with pESC-At Cyt b_5 and Cyt b_5 reductase (EPX + b_5, $b_5$red). The means represent five replications and the detection limit for Est and Va were ~0.05% of total fatty acids in this experiment.

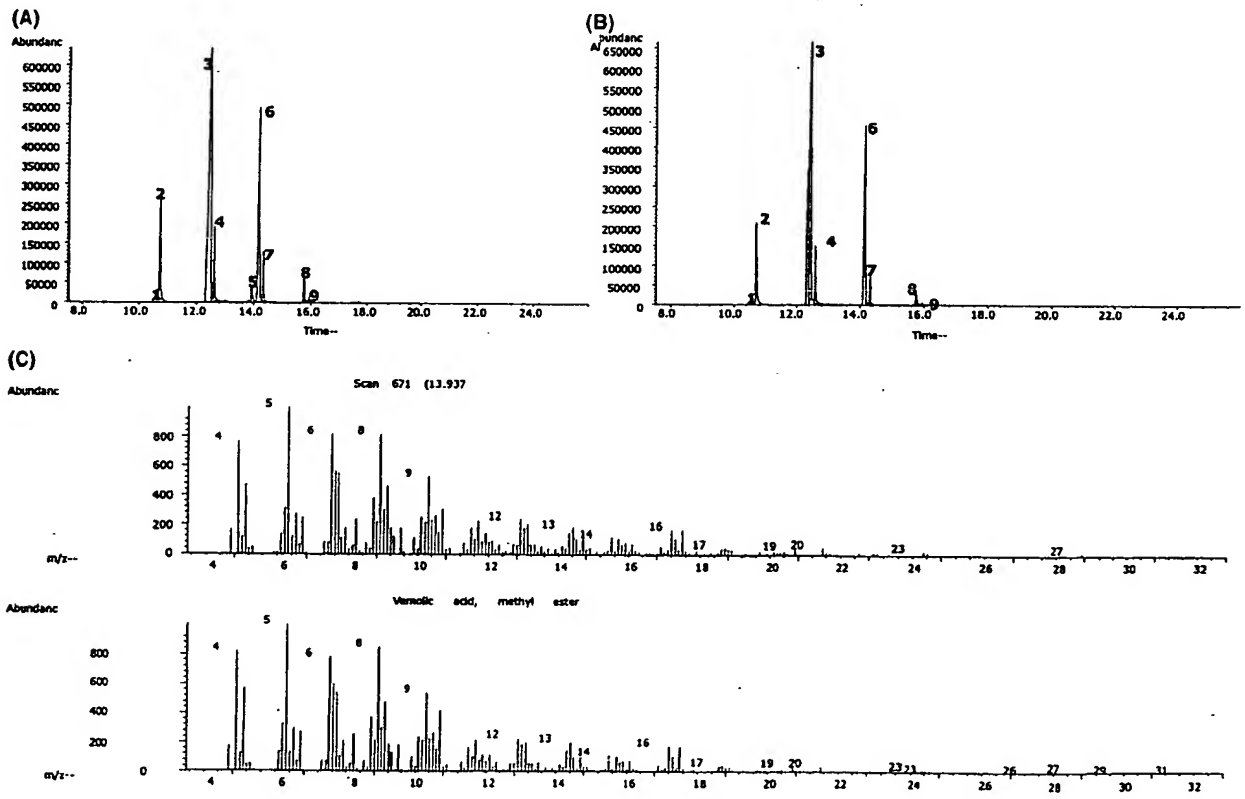

Fig. 4. GC–MS analysis of fatty acid derivatives from transgenic *Arabidopsis* seeds. (A) Chromatograms from *Arabidopsis* transformed with pCAMBIA1201 inserted with the cDNA from *S. laevis*. (B) Chromatograms from *Arabidopsis* transformed with empty vector, pCAMBIA1201 as a control. (C) Mass spectrum of the compound giving rise to peak 5 at 13.94 min in chromatogram (A), and a standard vernolic acid 2. *m/z*, mass-to-charge ratio. Peak 1, 7-hexadecenoic acid; 2, hexadecanoic acid; 3, 9-octadecenoic acid; 4, octadecanoic acid; 6, 11-eicosenoic acid; 7, eicosanoic acid; 8, docosenoic acid; 9, docosanoic acid.

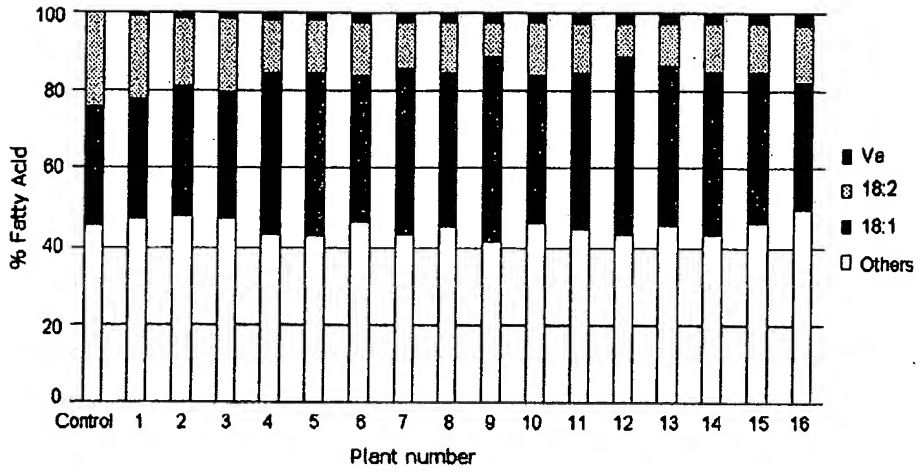

Fig. 5. Seed oil fatty acid profile for transgenic *Arabidopsis thaliana* carrying the *S. laevis* epoxygenase. Control (CTRL) seeds of vector transformed T_2 *Arabidopsis* seeds = the average of two replications. Plants 1–16 are T_2 seed analyses of different epoxygenase transgenic plants. *Va = vernolic acid.

vernolic acid **2** were 1.3–10.6 μg/mg DW, the average was 5.8 μg/mg DW, and their percentages of vernolic acid **2** in the total fatty acids were 1.1–3.1%, the average value was 2.4%.

Singh et al. (2001) reported that a very marked increase in oleic acid (18:1) and decrease in linoleic (18:2) and linolenic (18:3) acids in *Arabidopsis* plants transformed with the *C. palaestina* epoxygenase gene. The same trend is found in our results (Fig. 5), this indicates endogenous Δ^{12} desaturation was reduced in these transgenic plants.

2.4. Analysis of epoxygenase mRNA expression

The presence of the epoxygenase gene transcripts in various *S. laevis* tissues was analyzed by RNA blot analysis with the cloned cDNA as a probe (Fig. 6). The transcript expressed strongly in developing embryos and slightly in mature embryos but it was not detected in leaves, or roots. This is not surprising since epoxy fatty acids only accumulate in oil of developing seeds but this is the first confirmation of this fact of desaturase-like epoxygenases. Previously Cahoon et al. (2002) reported that the gene encoding a cytochrome P450 epoxygenase enzyme from *E. lagascae*, CYP726A1, is expressed in developing seeds but is not detectable in leaves. The presence of epoxygenase transcript in mature seeds is likely due to incompletely turned-over RNA remaining from expression earlier in seed development.

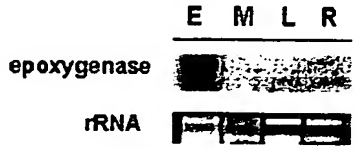

Fig. 6. RNA blot analysis of the *Stokesia* epoxygenase gene in different *Stokesia* plant tissues. A DIG-labeled probe corresponding to the full length *Stokesia* epoxygenase gene was hybridized to 5.5 μg of total RNA from developing embryos (E), mature seeds (M), leaves (L) and roots (R).

3. Experimental

3.1. cDNA cloning

A partial *S. laevis* epoxygenase cDNA fragment was obtained from RNA of developing embryos of *S. laevis* using an Access RT-PCR System (Promega Co.). The PCR mixtures contained 1 μg of total RNA template, 0.2 mM dNTPs, 2.5 U of AMV reverse transcriptase, 2.5 U of *Tfl* polymerase and 1 μM each of two degenerate primers described below. Reaction mixtures were incubated in a thermocycler (Perkin–Elmer, Model 2400) for 45 min at 48 °C, followed by 2 min at 94 °C and 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C. The PCR primers used (5epoxy, 5′-GGICAYGARTGYGGNCAYCAYGC-3′ and 3epoxy, 5′-ACRTGIGTRTGNGTNACRTCRTG-3′) represent two peptide sequences, [GHECGHHA] and [HDVTHTHV], which are the conserved regions in amino acid sequences of desaturase-like epoxygenases of *Crepis palaestina* and *V. galamensis*. The amplified products of ~S620 bp were fractionated on a 1% agarose gel, extracted from the gel using Quiaquick Gel Extraction Kit (Qiagen Inc.) and subcloned into the pGEM-T Easy vector (Promega Co.). The DNA inserted was sequenced in both directions.

For determination of the full-length cDNA sequence, a Rapid Amplification of cDNA Ends (RACE) strategy was applied to obtain the 5′ and 3′ ends beyond the internal fragment cloned with the internal primers

above. A cDNA was synthesized from poly(A)+RNA of developing seeds of *S. laevis* using a Marathon cDNA Amplification Kit (BD Biosciences Clontech). We then designed the following two primers from the sequence information of the partial cDNA fragment of *S. laevis* epoxygenase; 5′-ST: 5′-CGCAACCTGGATTCGCTCACGCTCGG-3′, and 3′-ST: 5′-CCCAGCTCAGGACTTACTCCACATACG-3′. The 5′-half and 3′-half of the cDNAs were amplified using the PCR conditions described in the user manual of the kit. Fractionation of the amplified fragments, cloning and sequencing were carried out as described above.

The deduced amino acid sequence similarities were determined by a software, Lasergene (DNASTAR Inc.). A phylogenic tree was drawn using 'SEQ Web, version 1.1' (Genetics Computer Group Inc.).

3.2. Gene testing in yeast

For expression in yeast, two different approaches were applied. At first, a *Bam*HI site was introduced at the first ATG and *Bsm*BI and *Eco*RI sites were introduced at the end of the open reading frame or coding sequence (ORF) of the cDNA by PCR mutagenesis. The ORF sequence of the cDNA was amplified using two primers (StexpF1: 5′-ACGCGGATCCATGTCGGATTCATATGATG-3′, StexpR1: 5′-GACGCGTCTC*GAATTC*TACATTTTATGGTACCAATATG-3′, *Bam*HI and *Bsm*BI sites are underlined, *Eco*RI site is in italic), and cloned into the pGEM-T Easy vector and verified by DNA sequencing. The *Bam*HI–*Bsm*BI fragment covering the entire ORF of the cDNA was cut out from pGEM-R Easy vector and ultimately cloned into the respective sites of the expression vector, pYeDP60. The yeast strain INVSc1 was transformed with the vector, harboring either no insert or the cDNA. The transformed yeasts were cultured in expression media with or without 1 μM linoleic acid for two days and the cells were collected. Their lipids were extracted with chloroform:methanol (2:1), methylated with diazomethane and sodium methoxide, and the methyl ester fatty acids were analyzed with gas chromatography–mass spectrometry.

For the second approach, the different plasmid vectors pYES2 (Invitrogen, Carlsbad, CA) and pESC (Stratagene, La Jolla, CA), were used to transform the yeast strain INVSc1. Both plasmids contain an *Escherichia coli* replication origin, a yeast 2m plasmid replication origin, an *E. coli* ampicillin-resistance gene and the yeast *URA3* gene in pYES2 and *TRP1* gene in pESC. The pYES2 carries the promoter and enhancer sequence from the *GAL1* gene for regulated expression. On the other hand, the pESC contains the *GAL1* and *GAL10* yeast promoters in opposing orientation.

To study the *Stokesia* epoxygenase gene function in yeast, a full-length cDNA fragment was cloned into pYES2 to give St EPX-pYES2. pYES2 together with pESC as a vector control (CTR), St EPX-pYES2 together with pESC (EPX) were transformed into yeast cell and selected on yeast minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose).

The total lipid composition of yeast was determined from cells harvested from a 3 ml liquid culture according to slightly modified the method described by Cahoon et al. (2002). Yeast CTR and EPX, bred colonies containing the pYES2 and pESC expression plasmids with or without St EPX cDNA were grown for three days at 30 °C in media (Cahoon et al., 2002) lacking uracil and tryptophan, and were supplemented with glycerol and glucose to final concentration of 5% (v/v) and 0.5% (w/v), respectively. Cells were washed twice in fresh media (Cahoon et al., 2002). The washed cells were then diluted to OD_{600} = 0.4 in media consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) galactose, 0.01% (w/v) adenine, and 0.2% (w/v) Tergitol NP-40. The media was supplemented with linoleic acid **1** at a final concentration of 470 nM. The cultures were incubated with shaking at 250 rpm at 20 °C and grown to OD_{600} = 12. Cells were collected by centrifugation and washed with sterilized water and pelleted cells were freeze-dried. Fatty acid methyl esters were prepared by transesterification of the dried cell pellet in 1% (w/v) sodium methoxide in methanol and analyzed using gas chromatography.

3.3. Gene testing in plants

For the *Arabidopsis thaliana* expression system, the TDNA-vector, pCAMBIA 1201 was utilized. A *Bsm*BI site was introduced at the first ATG and an *Sma*I site was introduced at the end of the ORF of the cDNA by PCR mutagenesis. The ORF sequence of the cDNA was amplified using two primers, (StexpF2: 5′-GACGCGTCTCCCATGTCGGATTCATATGATG- 3′, StexpR2: 5′-GACGCCCGGGTTACATTTTATGGTACCAATATGTCCC- 3′, *Bsm*BI and *Sma*I sites are underlined), and cloned into the pGEM-T Easy vector and verified by DNA sequencing. The *Bsm*BI–*Sma*I fragment covering the entire open reading frame of the cDNA was cut out from pGEM-T Easy vector and ultimately cloned into the respective sites of a vector, which contains a phaseolin promoter cassette (Kawagoe et al., 1994; Bustos et al., 1998). The *Pst*I fragment including the cDNA with the phaseolin cassette was cut out from the pPHI4752 vector and cloned into the respective multi-cloning site of pCAMBIA1201, T-DNA vector (CAMBIA, 2003).

This construct, pCAMBIA-ST was transformed into the *Agrobacterium tumefaciens* strain C58 harboring GV3850 vector by a triparental matings method. The original pCAMBIA1201 was also transformed into *Agrobacterium* as a control.

A. thaliana ecotype Columbia plants were transformed with the *Agrobacterium* carrying pCAMBIA-ST or pCAMBIA1201 using a simplified dipping method (Clough and Bent, 1998). T_1 seeds were collected and cultured on selection media (MS salts, B5 vitamins, 1% sucrose, 25 mg/l hygromycin, 500 mg/l cefotaxime and 0.8% Phytagar, pH 5.8). Surviving plantlets were transferred into soil and T2 seeds were collected. For lipid extraction, seeds were ground in chloroform–methanol (2:1). The extracts were brought to dryness under N_2 gas stream. The lipid residues were immediately dissolved in a few drops of diazomethane and 0.5 ml 1% (w/v) sodium methoxide solution and shaken for 45 min at room temperature. The methyl ester fatty acids were extracted in hexane. The samples were analyzed using GC–MS or GC (Dahmer et al., 1989).

3.4. Analysis of epoxygenase mRNA expression in different tissues

RNA was isolated from each tissue such as developing seeds, leaves and roots using the Trizol reagent as described by the manufacturer (Invitrogen) and from mature seeds as described by Naito et al. (1994). RNA was separated on a 1% denaturing formaldehyde gel and transferred onto a Zeta-Probe Blotting Membrane (Bio-Rad Laboratories) according to Chomczynski (1992). Equalized loading of RNA was checked by ethidium bromide staining of rRNAs. The entire coding region of the epoxygenase gene from *S. laevis* was labeled with digoxigenin (DIG)-UTP by a PCR DIG Probe Synthesis Kit (Roche Applied Science). The hybridization was performed at 65 °C overnight in 1 mM EDTA, 0.5 M Na_2HPO_4 (pH 7.2) and 7% SDS. The final washing step were performed at 65 °C in 1 mM EDTA, 40 mM Na_2HPO_4 and 2% SDS. Hybridized mRNAs were detected with alkaline phosphatase conjugated anti-DIG antibody (Roche Applied Science) and its chemiluminescent substrate, CDP-Star (Roche Applied Science)

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Acknowledgements

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